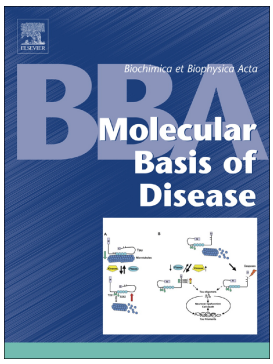
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PII: S0925-4439(20)30376-8

DOI: https://doi.org/10.1016/j.bbadis.2020.166028

Reference: BBADIS 166028

To appear in: *BBA - Molecular Basis of Disease*

Received date: 16 October 2020

Accepted date: 18 November 2020

Please cite this article as: Y. Pedra-Rezende, M.C. Fernandes, C. Mesquita-Rodrigues, et al., Starvation and pH stress conditions induced mitochondrial dysfunction, ROS production and autophagy in Trypanosoma cruzi epimastigotes, *BBA - Molecular Basis of Disease* (2020), https://doi.org/10.1016/j.bbadis.2020.166028

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Starvation and pH stress conditions induced mitochondrial dysfunction, ROS production and autophagy in *Trypanosoma cruzi* epimastigotes

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Abbreviations

3-Methyladenine (3-MA); Acetyl coenzyme A (acetyl-CoA); Antimycin A (AA); Autophagy-related proteins (Atgs); Carbonyl cyanide 4(trifluoromethoxy)phenylhydrazone (FCCP); 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB); Fetal bovine serum (FBS); Horseradish peroxidase (HRP); Hydrogen peroxide (H_2O_2); Mitochondrial electron transport system (ETS); Monodansyl cadaverine (MDC); N-acetylcysteine (NAC); Oxidative phosphorylation (OXPHOS); Potassium cyanide (KCN) ; Reactive oxygen species (ROS); Tetramethylrhodamine (TMRE); Triatominae artificial urine (TAU); Trypanothione reductase (TR); Tryparedoxin peroxidase (TrypP).

ABSTRACT

Chagas disease is a neglected illness endemic in Latin America that mainly affects rural populations. The etiological agent of Chagas disease is the protozoan *Trypanosoma cruzi*, which has three different parasite stages and a dixenous life cycle that includes colonization of the vertebrate and invertebrate hosts. During its life cycle, *T. cruzi* is subjected to stress conditions, including variations in nutrient availability and pH, which impact parasite survival and differentiation. The plasticity of mitochondrial function in trypanosomatids has been defined as mitochondrial activity related to substrate availability. Thus, mitochondrial remodeling and autophagy, which is a constitutive cellular process of turnover and recycling of cellular components, may constitute a response to the nutritional and pH stress in the host. To assess these processes, epimastigotes were subjected to acidic, alkaline, and nutritional stress conditions and mitochondrial function and its influence on the autophagic process were evaluated. Our data demonstrated that the three stress conditions affected the mitochondrial structure, inducing organelle swelling and impaired oxidative phosphorylation. Stressed epimastigotes produced increased ROS levels and overexpressed antioxidant enzymes. The stress conditions resulted in an increase in the number of autophagosomes and exacerbated the expression of different autophagy-related genes (Atgs). A correlation between mitochondrial dysfunction and autophagic phenotypes was also observed. After 24 h, acid stress and nutritional deprivation induced metacyclogenesis phenotypes (mitochondrial remodeling and autophagy). On the other hand, alkaline stress was transient due to insect blood feeding and culminated in an increase in autophagic flux as a survival mechanism.

Keywords: *Trypanosoma cruzi*; mitochondria; pH stress; nutritional deprivation; autophagy.

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1. Introduction

Trypanosoma cruzi is a protozoan parasite that causes Chagas disease, a neglected tropical disease affecting millions of individuals. Despite successful control interventions, this illness remains an important public health problem especially for low-income people in Latin America [1–3]. In endemic countries, classical transmission of the disease is based on contamination through the vector excretions; however, new cases are mainly associated with congenital or oral infections [4,5]. In (a) wild environment(s), the oral route is the most frequent recurrent transmission mechanism and becomes clinically significant due to deforestation [6]. In Brazil, relevant percentage of açaí and derivatives containing *T. cruzi* DNA have been detected in various geographic areas [7].

Chagas disease has acute and chronic phases with clearly different clinical manifestations. Cardiac and/or digestive complications can lead to loss of work productivity of chronic individuals causing a massive economic impact of billions of dollars [8]. Current chemotherapy is still limited to nitroderivatives benznidazole or nifurtimox, which have been available for half a century, have limited activity (especially in the chronic phase) and cause important adverse effects [9]. These limitations have motivated multidisciplinary initiatives to develop novel drugs and/or drug combinations for preclinical and clinical trials [10].

The *T. cruzi* life cycle is considered complex and involves vertebrate and invertebrate hosts and at least three parasite stages [11]. After triatominae blood feeding, metacyclic trypomastigotes present in the vector excretions reach the mammalian bloodstream and can infect all nucleated cells. In an intracellular environment, the parasites are subjected to acid stress that triggers their differentiation to amastigotes, a proliferative form. After proliferation, amastigotes differentiate into the bloodstream trypomastigotes that promote mechanical lysis of the host cell thus disseminating the infection to new tissues or cells. Uninfected

triatomines ingest the blood containing trypomastigotes, which reach the midgut and differentiate into epimastigotes that adhere to the epithelium and proliferate. Several replicative rounds take place before the parasites migrate to the insect rectum, where the differentiation takes place. This process is known as metacyclogenesis and occurs due to peculiar environmental conditions, such as acid pH and nutritional deprivation [11].

Autophagy is a constitutive process of turnover and recycling of damaged or redundant cellular components [12]. This pathway is tightly regulated by autophagy-related proteins (Atgs), and Atg8 expression is extensively used for monitoring of autophagic flux [13]. Morphological evidence of autophagy have been described in trypanosomatids since 1970s, especially in parasites subjected to stress conditions [14–17]. Atgs homologs were identified in these protozoa; however, numerous components are absent (e.g., the components of the Atg12 conjugation system) [18,19]. Bioinformatics approaches indicate that the Atg8 conjugation system appears to be relatively similar to the mammalian system [18], and the biological relevance of the autophagic pathway was postulated in *T. cruzi* by Alvarez and colleagues. This group demonstrated the role of autophagy during metacyclogenesis demonstrating that Atg8 is localized in autophagosomes [20]. The target of rapamycin (TOR) complexes and kinases were identified in the parasite genome; however, their biological functions are unknown [17,21]. In *T. brucei*, selective autophagy of glycosomes was reported, and the biological implications of the degradation of this organelle were associated with parasite bioenergetics [22,23].

Mitochondria are involved in aerobic ATP synthesis via oxidative phosphorylation (OXPHOS) and in the redox balance; these organelles are one of the major sources of reactive oxygen species (ROS). During respiration, consumed oxygen is incompletely reduced to water, and partial reduction generates superoxide radicals in the mitochondrial matrix that are subsequently

dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutases in the intermembrane space [24,25]. Various mechanisms of metabolic remodeling are used to avoid the deleterious effects of ROS in various biological and/or pathological situations. These mechanisms involve a reduction in the mitochondrial ATP synthesis or an increase in the mitochondrial membrane permeability leading to enhanced efficiency of reduction by OXPHOS [26]. The mitochondrial architecture of *T. cruzi* is singular, i.e., there is only a single ramified organelle per parasite with the kinetoplast region that contains all mitochondrial DNA. The parasite mitochondrion has been described as a promising target of novel drugs due to the absence of classical antioxidant defenses, including glutathione/glutathione reductase system and catalase, which indicates vulnerability of the mitochondrion to ROS [27]. Electron leakage from the mitochondrial electron transport system (ETS) is a critical event that generates high levels of ROS in *T. cruzi* [10,28]. It was postulated that differences in environmental conditions inside various hosts (variation in pH or temperature, availability of nutrients or substrates, etc.) induce metabolic adaptations of the parasite, such as modulation of ATP production [19].

The crosstalk of the mitochondrion and autophagic pathway has been described as a basis of the pathology of different diseases including Chagas disease [29,30]. Considerable efforts have been made to investigate mitochondrial physiology of *T. cruzi*; however, functional characteristics of the mitochondrion under stress conditions associated with complete biological cycle of the parasites are unknown, and the interactions between the mitochondrion and autophagy are poorly understood. Molecular mechanisms involved in this process can be critical for the success of the parasite in distinct environments inside the invertebrate and vertebrate hosts. Our hypothesis is that mitochondrial remodeling and autophagy may be a response to stress conditions during parasite metacyclogenesis. In this context, we analyzed mitochondrial functions and autophagic flux in epimastigotes

under pH and nutritional stress conditions by morphological, biochemical, and molecular approaches.

2. Materials and Methods

2.1. Reagents

3-MA, acetyl coenzyme A (acetyl-CoA), antimycin A (AA), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), cytochrome c from equine heart, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), horseradish peroxidase (HRP), MDC, NAC, oxaloacetate, saponin, sodium dithionite, and succinic acid were purchased from Sigma-Aldrich (St. Louis, USA). H_2O_2 and potassium cyanide (KCN) were from Merck (Darmstadt, Germany). Amplex Red and TMRE were obtained from Molecular Probes (Carlsbad, USA). TRIzol, secondary anti-rabbit antibody conjugated with Alexa Fluor 488, and a SuperScript Vilo kit were purchased from Invitrogen (Carlsbad, USA). Poly/Bed 812 resin was purchased from Polysciences, Inc. (Warrington, USA). A GE Healthcare illustraGFX PCR DNA and gel band purification kit, BigDye Terminator v3.1 cycle sequencing kit, and Pierce™ BCA protein assay kit were from Thermo Fisher Scientific (Waltham, USA). A GoScript kit and GoTaq® qPCR master mix were from (Promega, Madison, USA). Primary rabbit anti-TcAtg8 was from Rheabiotech (Campinas, Brazil), and FBS was from Cultilab (Campinas, Brazil). All other reagents were of analytical or superior grade.

2.2. Parasites and stress induction

T. cruzi proliferative epimastigotes (Y strain) were maintained at 28°C in liver infusion and tryptose medium (LIT) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and were harvested during the exponential growth phase. The following experimental conditions were used for stress induction: control (pH 7 + 10% FBS), low FBS (LoFBS, 0% FBS), acid stress (AcS, initial pH 3 + 10% FBS), and alkaline stress (AlkS, initial pH 10 + 10% FBS); samples were incubated in 24-

well microplates for 24 and 96 h at 28°C, and parasite viability was directly quantified.

2.3. Transmission electron microscopy analysis

After washing with phosphate-buffered saline (PBS, pH 7.2), stressed epimastigotes (5 x 10^6 cells/mL) were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 30 min and postfixed with 1% OsO_4, containing 0.8% potassium ferricyanide and 2.5 mM $CaCl_2$ in the same buffer for 20 min at room temperature. Subsequently, dehydration steps were performed in an ascending acetone series, and the samples were embedded in Poly/Bed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Analysis was performed using a JEM1011 transmission electron microscope (Jeol, Tokyo, Japan) located in Plataforma de Microscopia Eletrônica Rudolf Barth (Instituto Oswaldo Cruz, Fiocruz).

2.4. Flow cytometry analysis

Control and stressed epimastigotes were used in two flow cytometry assays. (a) Mitochondrial membrane potential ($\Delta\Psi m$) was assessed by labeling with 50 nM TMRE for 15 min; the changes in TMRE fluorescence were calculated as index of variation (IV) according to the equation (MT - MC)/MC, where MT is the median fluorescence of the stressed parasites and MC is the median fluorescence of the control parasites. Negative IV values represent the mitochondrial membrane depolarization. The addition of 10 µM FCCP dissipates $\Delta\Psi m$ and was used as a negative control. (b) ROS production was assayed after incubation with 10 µM DHE for 30 min; 22 µM (AA) was added as a positive control. The samples were incubated on ice until analysis, and the data were acquired using a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) equipped with the CellQuest software (Becton Dickinson). A total of 10,000 events were analyzed.

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2.5. High performance respirometry analysis

Control and stressed epimastigotes (2.5 x 10^7 cells/mL) were resuspended in 2 mL of respiration buffer (125 mM sucrose, 5 mM succinate, 65 mM KCl, 10 mM Tris-HCl pH 7.2, 1 mM $MgCl_2$, and 2.5 mM monobasic potassium phosphate) at 28°C for the analysis by a high performance Oxygraph-2K respirometer (Oroboros Instruments, Innsbruck, Austria). The oxygen flux and concentration were determined by the DatLab software, and 2 µM AA added to achieve residual oxygen consumption (ROX) as reported previously [31].

2.6. Enzymatic activities assays

For the enzymatic assays, control and stressed parasites (1 x 10^8 cells) were thawed and disrupted by sonication; all assays were performed in the homogenates as previously described [32]. Protein concentration was determined by a Pierce™ BCA protein assay kit (Thermo Fisher, Waltham, Massachusetts, EUA). To assay citrate synthase (CS) activity, the generation of reduced coenzyme A (CoA-SH) was determined in the reaction of 0.25 mM thiol-specific reagent DTNB, 0.03 mM acetyl-CoA and 0.5 mM oxaloacetate in 75 mM Tris-HCl (pH 8.0) buffer at 412 nm [33]. The analysis was performed at room temperature in a total reaction volume of 1 mL using a UV-2550 spectrophotometer (Shimadzu Corp., Kyoto, Japan). To evaluate activities of mitochondrial complexes, the AA-sensitive succinate:cytochrome c activity (complexes II–III) was monitored by ferricytochrome c reduction at 550 nm (ε=19 mM^{-1} cm^{-1}) in the reaction mixture containing 100 mM potassium phosphate buffer pH 7.4, 150 µM equine heart cytochrome c, 3 mM succinic acid and 1 mM KCN. The activity of KCN-sensitive cytochrome c oxidase (complex IV) was measured by ferrocytochrome c oxidation at 550 nm (ε=19 mM^{-1} cm^{-1}) in the reaction mixture containing 100 mM potassium phosphate buffer pH 7.4 and 50 µM sodium dithionite-reduced equine heart cytochrome c. Next, 2 µM AA (complex II–III) and 1 mM KCN (complex IV) were

added as the controls [31]. To assess trypanothione reductase activity, the samples were added to the reaction mixture (1 μM $T(SH)_2$, 150 μM NADPH, 400 mM HEPES pH 7.5, 25 μM DTNB, and 1 mM EDTA), and 2TNB production was measured at 412 nm [34]. Activities of the mitochondrial complexes and trypanothione reductase were assayed at room temperature in a total reaction volume of 200 μL in a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, USA).

2.7. Hydrogen peroxide production assay

Epimastigotes (5 x 10^8 cells/mL) were pretreated with 10 mM 3-MA for 2 h before stress induction. Control and stressed parasites were incubated with 5 μM Amplex Red and 200 μg/mL HRP in respiration buffer [35]. The generation of resorufin was analyzed in a SpectraMax M3 fluorimeter (excitation: 530 nm; emission: 590 nm).

2.8. Gene expression analysis

RNA of control and stressed epimastigotes (1 x 10^8 cells/mL) was extracted using TRIzol according to the manufacturer's instructions. Total RNA was quantified in a spectrophotometer, and cDNA was synthesized with a GoScript kit. Quantitative PCR (qPCR) reactions were performed using an ABI Prism 7500 FAST system (Applied Biosystems) with GoTaq® qPCR master mix; GAPDH was used as a reference gene. Primer sequences are shown in Table S1. The comparative ΔΔCt method was used to compare the changes in the gene expression levels. PCR reaction products were purified using a GE Healthcare illustra™ GFX™ PCR DNA and gel band purification kit, and the sequencing was performed with a BigDye Terminator v3.1 cycle sequencing kit in a 3730 DNA analyzer (Table S1).

2.9. Fluorescence microscopy analysis

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Control and stressed epimastigotes were labeled with 100 μM MDC for 1 h at 28°C. After washing in PBS, the parasites were fixed in 4% paraformaldehyde for 40 min at 25°C. Alternatively, the parasites were preincubated with 500 μM NAC for 2 h before the induction of nutritional and pH stresses. Then, epimastigotes were fixed, permeabilized (PBS + 1% bovine serum albumin + 0.1% saponin + 5% FBS) for 1 h at 25°C, and incubated with primary rabbit anti-TcAtg8 (dilution: 1:200) for 1 h at 25°C. After washing, the samples were incubated with a secondary anti-rabbit Alexa Fluor 488 antibody (dilution: 1:2,000) for 1 h at 25°C. To prepare the positive control samples, the parasites were starved in PBS for 6 h. All images were acquired by a Zeiss AxioObserver M1 microscope (Oberkochen, Germany), and the percentage of positive parasites and the number of autophagosomes per parasite were determined using the ImageJ software (Bethesda, Maryland, USA). The validation of anti-Atg8 antibody was performed by SDS-PAGE followed by mass spectrometry identification (data not shown).

2.10. Statistical analysis

Nonparametric Mann-Whitney test by the IBM SPSS 22.0 software (IBM Corporation, Armonk, New York, USA) was used; the threshold for significance was set at $p \leq 0.05$.

3. Results

3.1. Nutritional restriction and acid and alkaline stresses reduce epimastigote viability

Initially, epimastigotes were incubated in the LIT medium containing 10% fetal bovine serum (FBS) under acidic (initial pH 3) or alkaline (initial pH 10) conditions to evaluate the impact of pH stress on parasite viability. Although the parasites partially buffered LIT medium in 96 h to pH 5 and 8.4 in AcS and AlkS, respectively

(data not shown), both conditions induced significant loss of parasite viability at least in one time point (Fig. 1). Different phenotypes were observed under acidic and alkaline stresses; AcS caused a time-dependent reduction of 16.6 and 63.8% in parasite viability in 24 and 96 h, respectively; AlkS induced a strong decrease in viability only after 24 h (42.2%), and viability recovered in 96 h. The nutritional deprivation also induced time-dependent reduction by 25.3 and 76.2% in 24 and 96 h, respectively, which is similar to the effect of AcS.

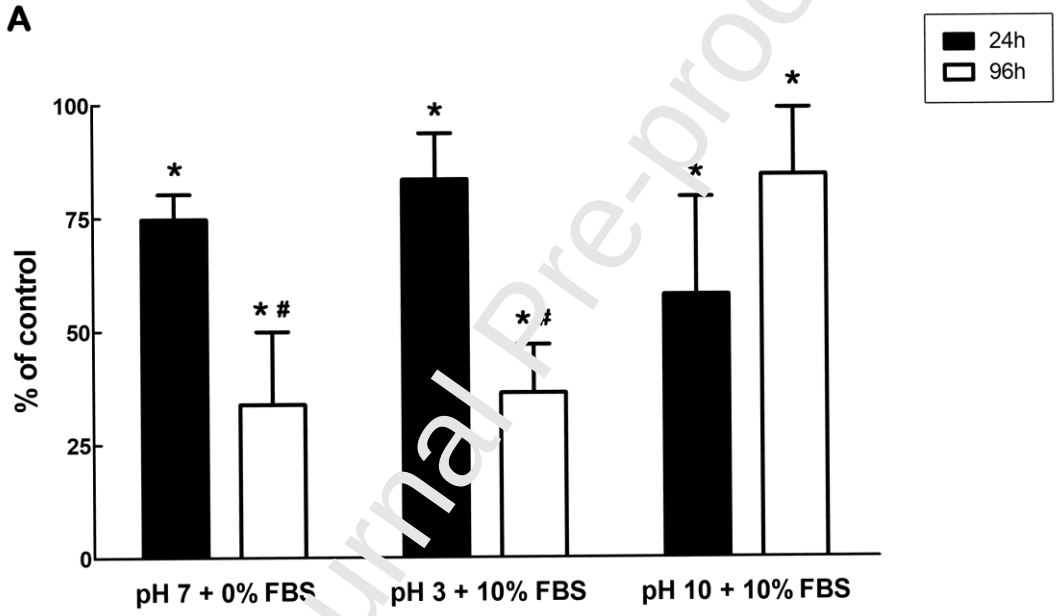


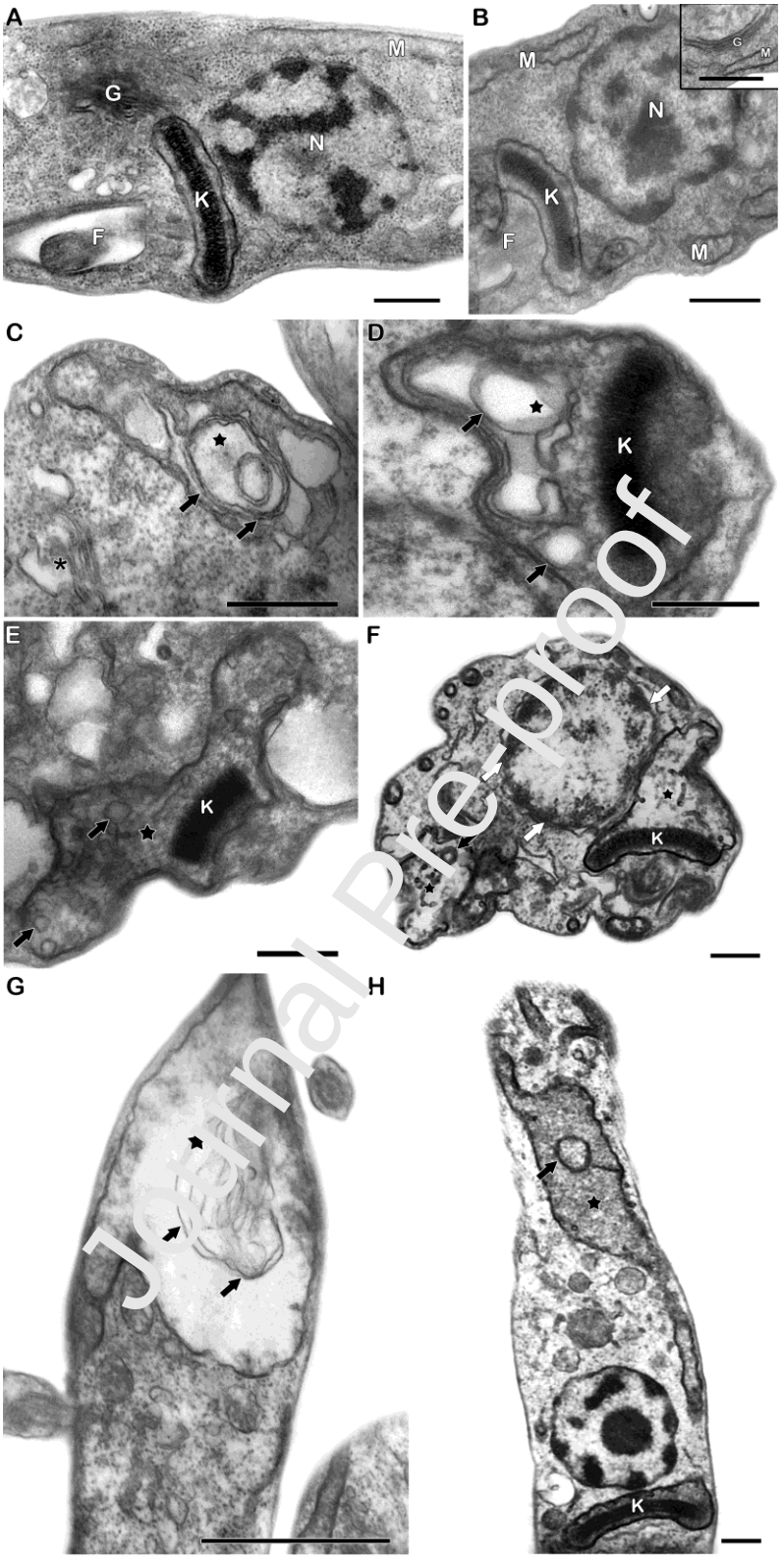
Fig. 1. Viability of *T. cruzi* epimastigotes is affected by FBS deprivation or acidification/alkalization of the culture medium. Parasite viability was determined by direct quantification and is expressed as % of control (pH 7 + 10% FBS). The graph shows mean ± s.d. of at least three independent experiments. *Significant differences versus control $p \leq 0.02$. #Significant differences between 24 and 96 h in the same experimental group $p \leq 0.05$ (Nonparametric Mann-Whitney test).

3.2. Mitochondrial swelling and autophagic features are the most frequent phenotypes in parasites under the nutritional and pH stress conditions

The effects of pH stress and nutritional deprivation on the ultrastructure of epimastigotes were also analyzed after 24 and 96 h. At both time points, the

control parasites maintained typical morphology of the cellular structures and organelles, such as elongated mitochondrion around the protozoan body, kinetoplast, nucleus, Golgi, and flagellum (Fig. 2A, B). LoFBS, AcS, and AlkS induced an intense mitochondrial swelling in 24 h characterized by washed-out matrix and loss of the inner membrane (Fig. 2C, E, G). These alterations persist after 96 h under three treatment conditions; nuclear damage and the dilatation of nuclear envelope was detected in the case of AcS (Fig. 2D, F, H). Inside the mitochondrion, concentric membrane structures were detected under all experimental conditions and at both time points (Fig. 2C-H').

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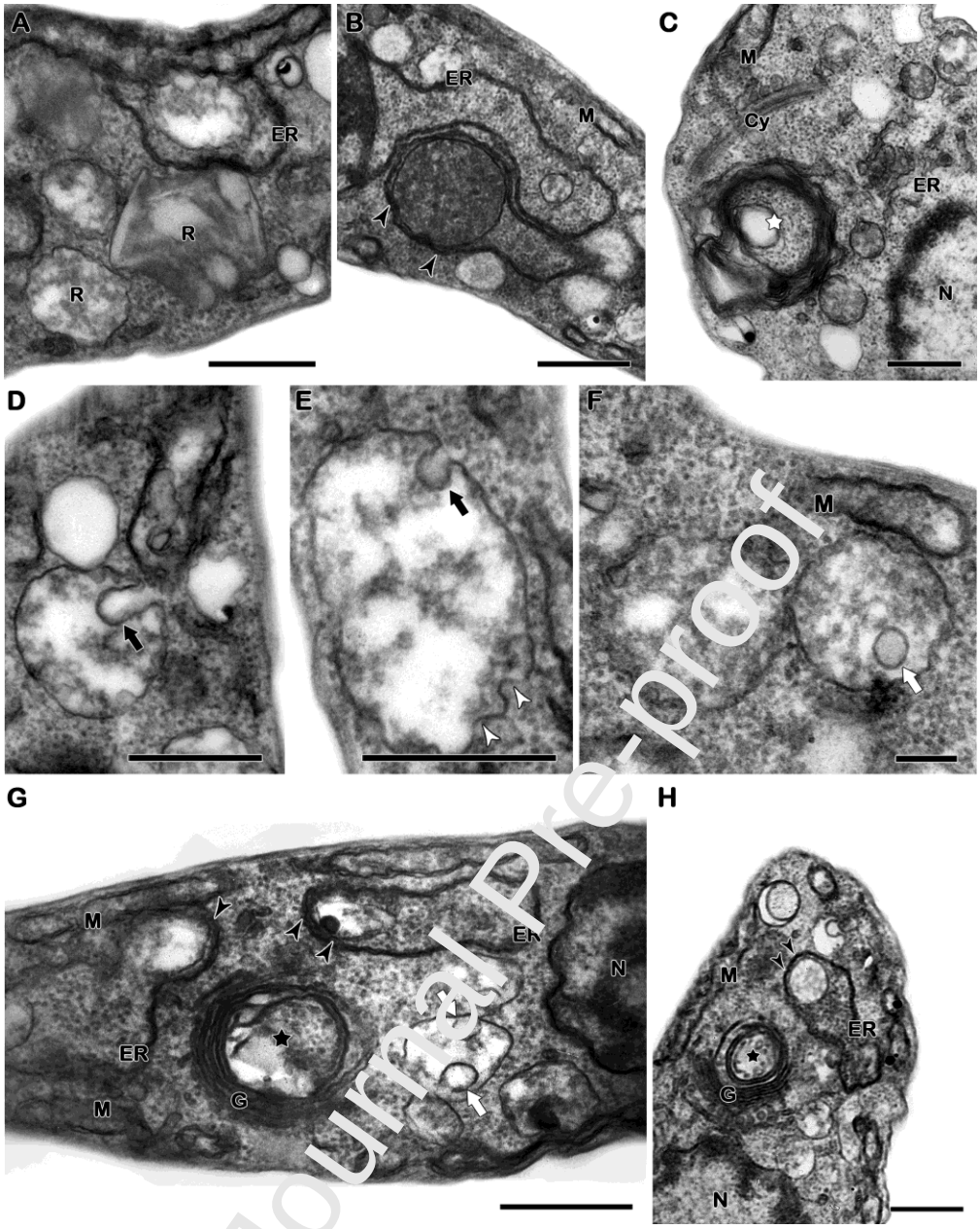
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Fig. 2. Mitochondrial swelling in *T. cruzi* epimastigotes under the nutritional and pH stress conditions. (A, B) Control parasites had typical morphology of epimastigotes. (C, D) LoFBS. (E, F) AcS. (G, H) AlkS. (C, E, G) 24 h. (D, F, H) 96 h. Nutritional and pH stresses induced mitochondrial swelling (black stars) with the presence of concentric membrane structures inside the organelle (black arrows). In

96 h, AcS induced a nuclear envelope dilation (white arrows). (N) nucleus, (K) kinetoplast, (M) mitochondrion, (G) Golgi, (F) flagellum. Bars in A-F and H = 0.5 µm; Bar in G = 0.2 µm.

Additionally, another important phenotype was, in parasites under all stress conditions in 24 and 96 h, in which the endoplasmic reticulum outline surrounded or was in close contact with cellular cytosolic vesicles and organelles (Fig. 3A-C, G, H). The appearance of Golgi outline surrounding portions of the cytosol was also observed in AcS and AlkS (Fig. 3G,H). After 96 h of nutritional deprivation, various stages of invagination of reservosome membrane were also detected, and small vesicles appeared inside the reservosome (Fig. 3D-F). Although the initial steps of reservosome invagination were not detected, small vesicles inside this organelle were detected after 96 h of acid stress (Fig. 3G).

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Fig. 3. Autophagic phenotype of *T. cruzi* epimastigotes under the nutritional and pH stress conditions. (A, D-F) LoFBS. (B, G) AcS. (C, H) AlkS. (A-C) 24 h. (D-H) 96 h. Nutritional deprivation and pH stresses induced a prominent autophagic phenotype with endoplasmic reticulum profiles (ER) surrounding and/or in close contact with various cellular structures (black arrowheads). In 96 h, LoFBS induced a recurrent invagination of the reservosome membrane observed during the initial (white arrowheads) and late (black arrows) stages of the process; some vesicles were detected inside the organelle (white arrow). At this time point, Golgi profiles surround cytosolic regions (black stars) in parasites in the AcS and AlkS groups. (N) nucleus, (M) mitochondrion, (G) Golgi, (R) reservosomes. Bars = 0.5 µm.

3.3. Nutritional and pH stresses impair mitochondrial OXPHOS but not CS activity

Mitochondrial functionality in stressed parasites was assessed by OXPHOS monitoring. Analysis of $\Delta\Psi m$ showed that parasites subjected to both types of pH stress manifest a decrease in Tetramethylrhodamine (TMRE) labeling from 53 to 76% in 24 and 96 h (Table 1). In contrast, LoFBS did not induce significant changes in TMRE labeling. Oxygen uptake was compromised under three stress conditions in 24 h (from 41.1 to 67.4% reduction) (Fig. 4A). In 96 h, this decrease was maintained in LoFBS (from 59.6 to 52.5%), partially reverted in AlkS (from 67.4 to 30.7%), and totally reverted in AcS (Fig. 4B).

Analysis of complex II-III demonstrated that incubation in the alkaline medium impaired the enzymatic activity at early and late time points (54.4 and 51.7% reduction, respectively), and the acidic condition reduced enzymatic activity only at 96 h (45.8%). Interestingly, nutritional deprivation resulted in a slightly different phenotype, in which a 2.3-fold increase was detected after 24 h followed by a reduction by 88.7% versus the control (Fig. 4C, D). Complex IV activity was upregulated in AcS at both time points (2.9- and 2.3-fold at 24 and 96 h, respectively), and AlkS and LoFBS increase the enzymatic activity in 24 h (1.7-fold) and 96 h (2.3-fold), respectively (Fig. 4E, F).

Quantitative PCR (qPCR) analysis demonstrated an increase in the expression of various ETS genes in the parasites under all stress conditions and at both time points (Fig. S1). After 24 h, AlkS induced the highest number of upregulated genes with a remarkable increase in the expression of CS (10.2-fold), complexes II (2.5-fold) and III (4.0-fold), and ATP synthase (2.9-fold); AcS and LoFBS induced upregulation of CS (4.3-fold) and complex III (1.3-fold), respectively (Fig. S1A). After 96 h, most of the upregulated genes remained overexpressed, including CS (1.9-fold) and complexes II (2.2-fold) and IV (3.3-fold)

in AlkS, CS (2.5-fold) in AcS, and complex III (1.6-fold) in LoFBS. Additionally, others genes were upregulated after long incubation, including complex IV (3.3-fold) in AlkS and CS (1.1-fold), complex IV (1.6-fold), and ATP synthase (2.0-fold) in LoFBS (Fig. S1B).

To determine whether the mitochondrial genes are modulated in a time-dependent manner, the data obtained after 24 and 96 h were compared. The prolonged exposure to alkaline stress increases the expression of complex IV (from 1.2- to 3.3-fold) and decreases the expression of CS (from 10.2- to 1.9-fold), complex III (from 4.0- to 1.1-fold), and ATP synthase (from 2.9- to 1.1-fold); these genes remain upregulated versus those in parasites treated under normal physiological conditions. Continuous exposure to nutritional deprivation modulates the gene expression in a time-dependent manner: the expression levels of CS (from 0.9- to 1.2-fold), complex IV (from 0.8- to 1.6-fold), and ATP synthase (from 1.4- to 1.9-fold) were increased after 96 h. CS is overexpressed under three stress conditions, and is the only gene modulated under the acidic conditions at both time points. To determine the importance of this enzyme in pH stress and nutritional deprivation, the enzymatic activity was assayed. Interestingly, although CS is overexpressed under all conditions, a decrease in citrate synthase activity by 64.5% was detected only in AcS in 24 h (Fig. 4G, H).

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Table 1. Δψm analysis in *T. cruzi* epimastigotes under nutritional and pH stresses

	24 h		96 h	
	Median	**IV[a]**	**Median**	**IV**
Control	46.2 ± 11.7 [b]	0.00	35.7 ± 16.0	0.00
Control + 10 µM FCCP	14.5 ± 7.1*	-0.73	12.9 ± 9.6*	-0.70
LoFBS	56.7 ± 47.6	-0.03	64.5 ± 18.5	0.40
AcS	15.6 ± 6.6*	-0.75	11.0 ± 3.4*	-0.76
AlkS	21.6 ± 9.5*	-0.53	19.4 ± 13.6*	-0.65

[a] IV = (ME – MC)/MC, were ME corresponds to the median fluorescence for parasites in experimental conditions, and MC corresponds to that of control parasites.

[b] Mean ± s.d. of 5 independent experiments.

* Significant differences in relation to the control group ($p \leq 0.05$).

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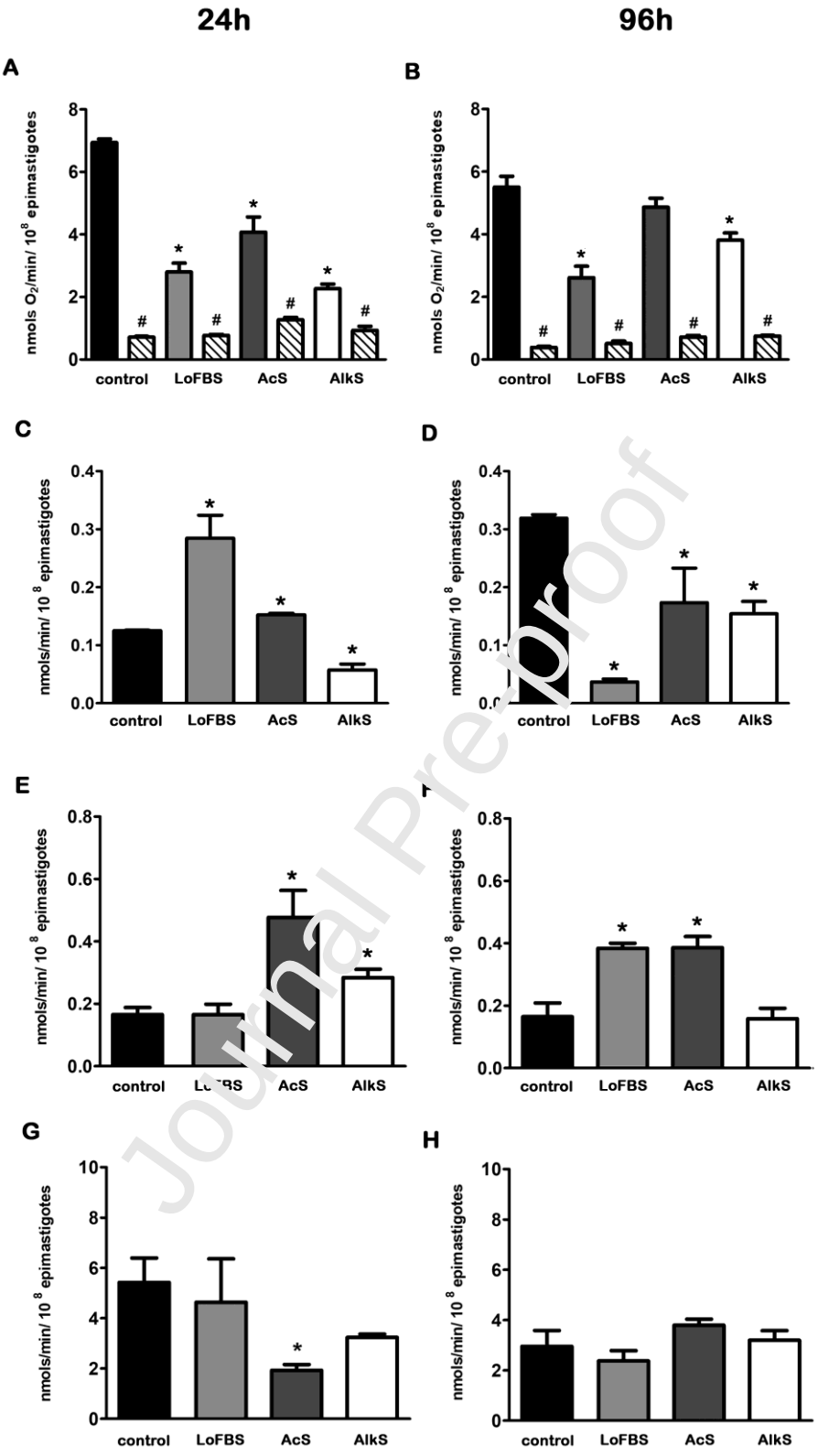


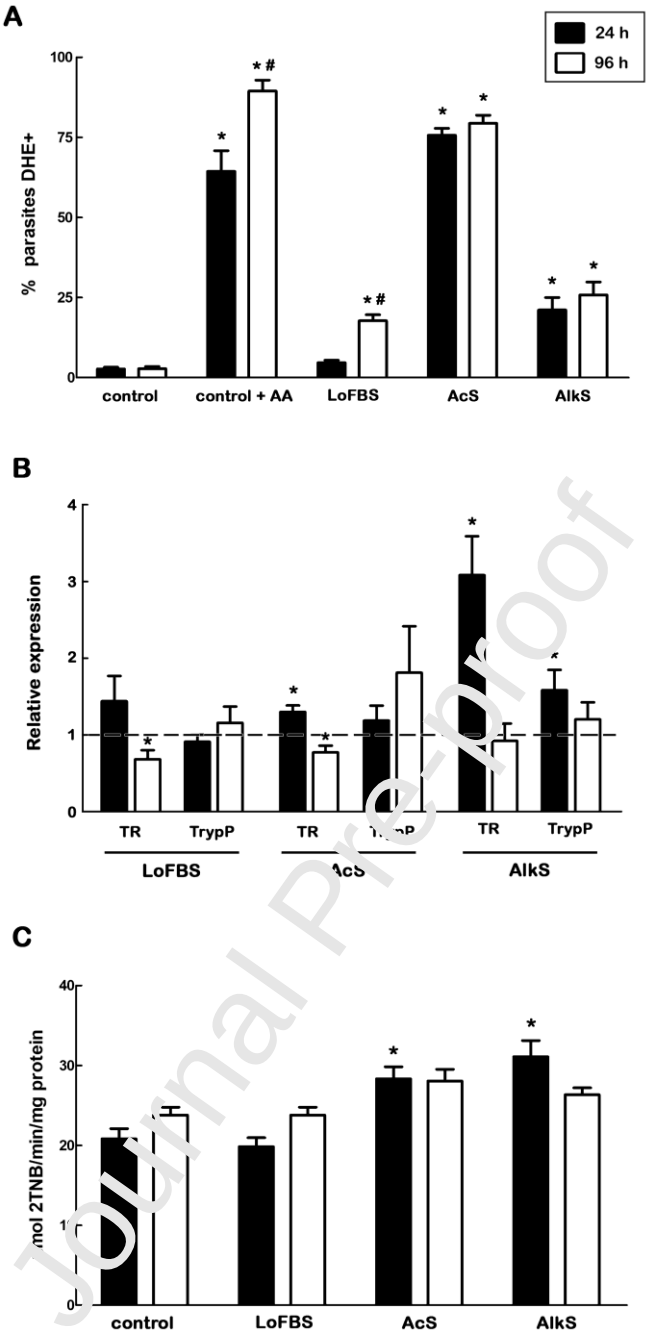
Fig. 4. Assays of OXPHOS and CS activity in *T. cruzi* epimastigotes under the nutritional and pH stress conditions. (A,B) Control (open bars) and ROX (dashed bars) states of O_2 consumption in stressed parasites. ROX state was induced by AA addition under all experimental conditions to confirm the mitochondrial

consumption. (C-F) The activities of the mitochondrial complexes were assayed in epimastigote homogenates; complex II-III activities (C,D) were determined as the rate of AA-sensitive ferricytochrome c reduction at 550 nm, and complex IV activity (E,F) was determined as the rate of KCN-sensitive oxidation at 550 nm. (G,H) CS activity was determined as the rate of DTNB reduction at 412 nm. The graphs show the mean ± s.d. of at least three independent experiments. *Significant differences versus control $p \leq 0.05$. #Significant differences between the control and ROX states in the same experimental group $p \leq 0.05$ (Nonparametric Mann-Whitney test).

3.4. ROS production and antioxidant defenses are induced in parasites under the nutritional and pH stress conditions

ROS production and antioxidant system were evaluated because they are an important aspect of mitochondrial physiology. DHE assay indicated that three conditions increase the percentage of epimastigotes generating ROS. AcS induced the highest number of labeled epimastigotes already in 24 h (75%), and the numbers remain constant until 96 h (80%). Similar phenotype was detected in AlkS in 24 h (20%) and 96 h (26%). Although nutritional deprivation does not trigger oxidative stress in 24 h, the percentage of labeled parasites is increased to 17.7% in 96 h (Fig. 5A). qPCR analysis demonstrated an increase in trypanothione reductase (TR) and tryparedoxin peroxidase (TrypP) expression in parasites under alkaline stress condition (3.1-fold and 1.6-fold, respectively) in 24 h. at the same time point, TR gene was also upregulated in AcS (1.3-fold). At late incubation time point, AcS and LoFBS induce a decrease in TR gene expression (32 and 23%, respectively) (Fig. 5B). In agreement with the qPCR analysis, TR activity was higher in AcS (36%) and AlkS (50%) in 24 h, and no increase was detected in 96 h (Fig. 5C).

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Fig. 5. ROS production and antioxidant defenses in *T. cruzi* epimastigotes under the nutritional and pH stress conditions. (A) Flow cytometry analysis of DHE labeling. (B) Gene expression analysis of antioxidant enzymes TR and TrypP normalized to the control transcript levels (black bar). GAPDH was used as an endogenous control. (C) TR activity calculated as 2TNB production assayed at 412 nm. The graphs in A and C show the mean and standard deviations of at least four independent experiments. The graph in B shows the mean ± s.e.m of at least three independent experiments. *Significant differences versus control $p \leq 0.05$.

[#]Significant differences between 24 and 96 h in the same experimental group $p \leq 0.05$ (Nonparametric Mann-Whitney test).

3.5. *Exacerbation of Atgs expression and increase in the number of autophagosomes in parasites under the nutritional and pH stress conditions*

Autophagy flux was assessed by qPCR of Atgs genes and quantification of autophagosomes and Atg8 expression by fluorescence microscopy. All stress conditions induced an increase in the relative expression of numerous Atg genes after 24 and 96 h. AlkS induced an increase in the expression of 7 autophagic genes after 24 h: Atg4 (2.2-fold), Atg8 (2.9-fold), Atg9 (3.0-fold), Atg15 (2.4-fold), Atg18 (4.5-fold), TOR1 (2.9-fold), and TOR2 (2.7-fold); AcS induced upregulation of Atg8 (2.9-fold) and TOR2 (2.5-fold), and LoFBS induced an increase in TOR1 (1.3-fold) at the same time point. Acid stress and nutritional deprivation induced downregulation of certain autophagic genes in 24 h, such as Atg15 and TOR1 (28.7 and 27.2%, respectively) in AcS and Atg3 and Atg7 (4.8 and 20.3%, respectively) in LoFBS (Fig. 6A).

In 96 h, the levels of most genes that were upregulated at an earlier time point by AlkS returned to the control levels; however, Atg4 (1.5-fold), Atg8 (2.0-fold), Atg9 (1.4-fold), and Atg18 (1.8-fold) remained overexpressed. At the same time point, Atg8 and Atg9 were upregulated under AcS and LoFBS conditions from 1.3- to 1.5-fold, and Atg18 (3.3-fold) and Atg3 (1.3-fold) were exclusively increased in AcS and LoFBS, respectively. The incubation under the acidic conditions for 96 h induced downregulation of Atg3 (9.5%) (Fig. 6B).

Comparison of the gene expression in 24 h and 96 h revealed a time-dependent upregulation of Atg18 (from 1.1- to 3.3-fold) in AcS and Atg3 (from 1.0- to 1.3-fold) and Atg7 (0.8-fold to 1.3-fold) in LoFBS. Conversely, a time-dependent

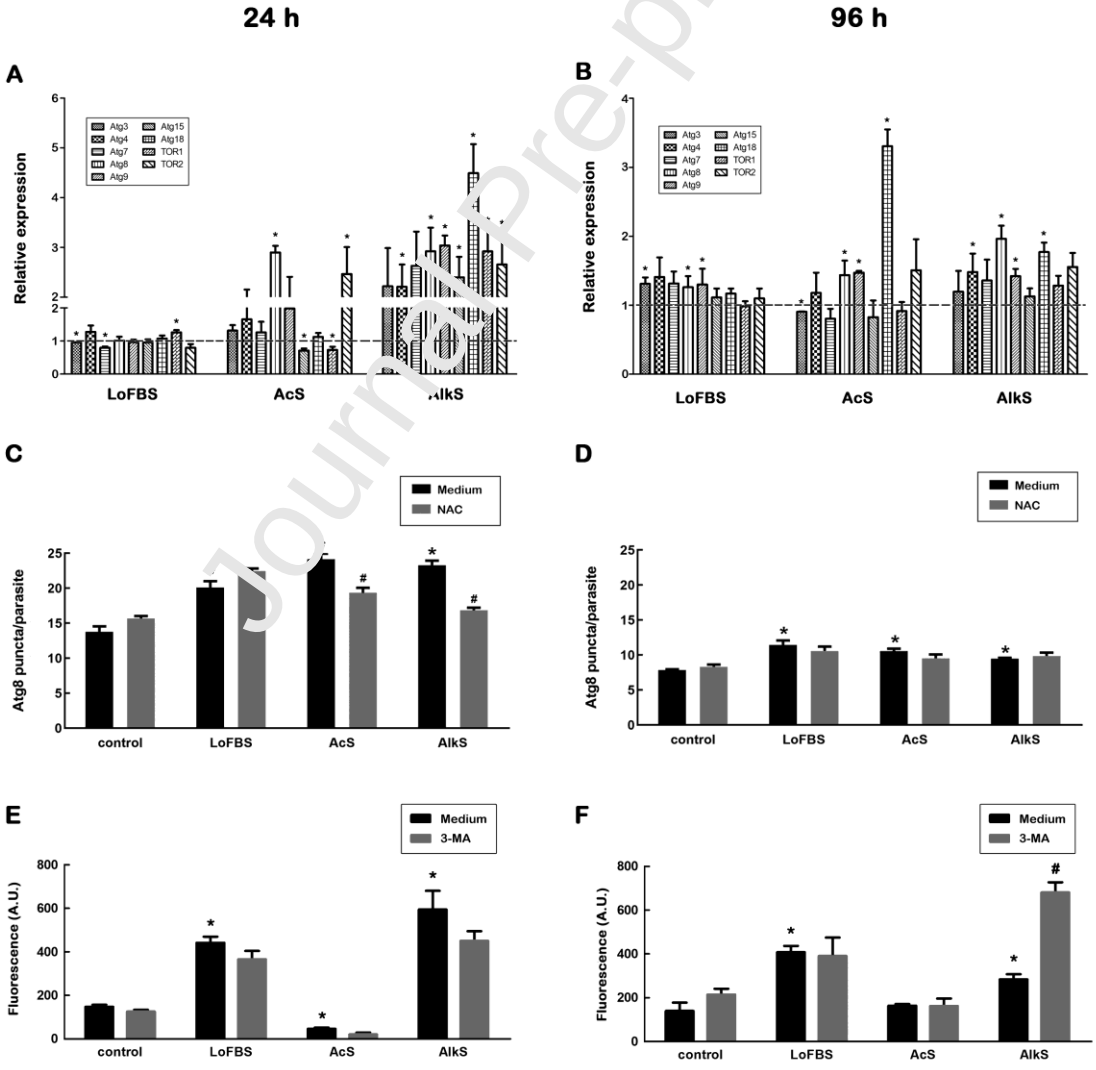
downregulation of the gene expression was observed for Atg3 (from 1.3- to 0.9-fold) and Atg8 (from 2.9- to 1.4-fold) in AcS and TOR1 (from 1.3- to 1.0-fold) in LoFBS. Interestingly, the incubation under AlkS conditions for 96 h also induced a reduction in the expression of 5 autophagic genes: Atg8 (from 2.9- to 1.9-fold), Atg9 (from 3.0- to 1.4-fold), Atg15 (from 2.4- to 1.1-fold), Atg18 (from 4.5- to 1.8-fold), and TOR1 (from 2.9- to 1.3-fold) compared to the levels detected after 24 h; however these genes remained upregulated compared to the levels in parasites under normal physiological conditions.

The results of Atg qPCR assay of Atg8 were consistent with the data of immunostaining, which indicated an increase in the autophagic pathway under three stress conditions. In 24 h, AcS and AlkS induced an increase in Atg8 puncta by 75 and 68%, respectively (Fig. 6C). Similarly, an increase in the Atg8 labeling after 96 h was detected in AcS (34%) and AlkS (20%) (Fig. 6D). Additionally, the number of Atg8 puncta was decreased in a time-dependent manner after long exposure to acidic and alkaline stresses. Although the expression of Atg8 gene increases only in 96 h, an increase in Atg8 puncta was detected already after 24 h (45%); this increase remains stable until 96 h (45%) (Fig. 6C, D). These data are confirmed by induction of an increase in the percentage of monodansyl cadaverine (MDC)+ parasites under three stress conditions (except LoFBS at 96 h) by up to 35%; this increase was more pronounced in AcS and AlkS (Fig. S2).

3.6. Mitochondrion dysfunction and ROS production lead to autophagy in parasites under pH stress conditions

To investigate a possible association between the mitochondrial dysfunction induced by pH stresses and nutrient deprivation, AtgAtg8 expression was assayed in parasites preincubated with an antioxidant, N-acetylcysteine (NAC). In parallel, ROS production was quantified in the presence or in the absence of an autophagy inhibitor, 3-Methyladenine (3-MA). In 24 h, preincubation with NAC reduced the

number of Atg8 puncta by 24 and 38% in AcS and AlkS, respectively. Interestingly, an increase in Atg8 puncta was detected in LoFBS; however, this phenotype was not suppressed by an antioxidant (Fig. 6C). The effect of NAC on the number of Atg8 puncta was not detected in 96 h (Fig. 6D); however, Atg8 labeling is decreased in all groups (including the control group) compared to the labeling detected in 24 h. At both time points, LoFBS (2.9-fold) and AlkS (from 4.0- to 2.0-fold) increase the H_2O_2 production according to the data of the Amplex red assay (Fig. 6E, F). Interestingly, pretreatment with 3-MA potentiates the oxidative effect of AlkS and increases H_2O_2 production 2.4-fold (Fig. 6F).



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Fig. 6. Detection of autophagy in *T. cruzi* epimastigotes under the nutritional and pH stress conditions. (A,B) The mRNA expression levels of Tor1, Tor2, Atg3, Atg4, Atg7, Atg8, Atg9, Atg15, and Atg18 under all experimental conditions were evaluated relative to the control condition (horizontal dot line). GAPDH was used as a reference gene. (C,D) Fluorescence microscopy analysis of Atg8 labeling. The graphs show the mean and standard errors of at least three independent experiments. (E,F) ROS production was measured by the fluorometric Amplex Red method. The graphs A and B show the mean ± s.e.m of at least three independent experiments. *Significant differences versus control $p \leq 0.05$. #Significant differences between the medium and NAC/3-MA treatments in the same experimental group $p \leq 0.05$ (Nonparametric Mann-Whitney test).

4. Discussion

During its life cycle, *T. cruzi* is challenged by environmental conditions that represent remarkable barriers to parasite infection in the host. In mammalian cells, trypomastigotes are subjected to acidification in the parasitophorous vacuole, which is one of the signals that triggers differentiation into replicative amastigotes [36]. In triatominae, epimastigotes are subjected to acidic environment that triggers metacyclogenesis in the insect rectum in combination with nutrient scarcity [37]. Interestingly, 24 h of the triatominae blood feeding, pH of insect excretion becomes alkaline (up to 8.9) and decreases time-dependently without a new blood ingestion [38]. Thus, the exposure of epimastigotes to alkaline conditions also occurs inside the vector. In this scenario, the molecular and biochemical strategies that promote *T. cruzi* survival during restriction of the nutrients and pH modulation (alkaline or acid) are essential for the maintenance of the parasite life cycle and are the focus of the present study.

LoFBS, AcS and AlkS conditions were used for all experiments to mimic the environment during epimastigote differentiation. Based on a preliminary kinetic analysis (data not shown), 24 and 96 h time points were selected to compare the initial and late effects of these stress conditions. Our data suggest that continuous

exposure of epimastigotes for extended time periods to medium acidification and FBS deprivation induce high mortality, which may represent an important limitation of the parasite population in the insect rectum. *In vitro*, metacyclogenesis is commonly induced by incubation of epimastigotes in triatominae artificial urine (TAU) medium, which is an acid phosphate-buffered solution (pH=5.0) [37,39].

Ultrastructural analysis indicates a pronounced impairment of the mitochondria and appearance of autophagic features under all stress conditions. Mitochondrial swelling and the presence of autophagosomes have been extensively described in *T. cruzi* under the unfavorable conditions [15,16,28]. Although all *T. cruzi* forms have a unique and branched mitochondrion [40], the mitochondrial metabolism undergoes considerable changes during life cycle of the parasite when distinct parasite forms are present in the environments with different nutrient availability [41]. Preliminary data from our group demonstrated different mitochondrial metabolism profiles in the bloodstream trypomastigotes and epimastigotes of *T. cruzi* suggesting a metabolic adaptation during the parasite cycle [31]. A mitochondrial dysfunction was detected in parasites subjected to alkaline stress at both time points; however, this condition may trigger a resistance mechanism in the parasites because a substantial increase in the expression levels of all mitochondrial target genes was detected in at least one of the evaluated time point; this observation is confirmed by a time-dependent increase in the complex II-III activity and the recovery of parasite viability after 96 h.

Interestingly, medium acidification and nutrient deprivation after 24 h causes metabolic modifications, especially in mitochondria; a combination of these changes results in a phenotype similar to that observed after differentiation of epimastigotes into trypomastigotes. These results are in agreement with the data of Gonçalves et al. [31] that demonstrated that bloodstream trypomastigotes have lower respiratory control ratio, Δψm, and citrate synthase activity suggesting mitochondrial impairment; however, the activity of complex II-III was higher compared with that in epimastigotes. Citrate synthase activity is a known indicator of the functional TCA cycle and

mitochondrial integrity; CS levels are usually reduced when glucose is available [42]. Low citrate synthase activity observed in AcS suggests that parasite is preparing to face high glucose levels present in the vertebrate host bloodstream. However, this phenotype is changed after 96 h, and the stressed parasites do not have features attributed to trypomastigotes. This shift may be due to separate evaluation of the stress conditions in the experiments, which did not provide full requirements for a complete metacyclogenesis [43]. An initial increase in the percentage of parasite death within the first hours of induction of metacyclogenesis *in vitro* [37] culminates in a reduction in the number of parasites that complete the differentiation. Interestingly, parasites exposed to acidic stress recovered in 96 h apparently in an attempt to reestablish the oxidative metabolism during the AcS challenge.

In eukaryotic cells, mitochondrial metabolism is one of the main sources of ROS [44]. Despite classical toxic effects of ROS [45], these oxidative species participate in several biological pathways, such as cellular signaling and proliferation [46,47]. Previous studies demonstrated that oxidative environment in the triatominae digestive tract after the insect blood meal triggers metacyclogenesis [47]; thus, stress conditions induce a considerable alterations in the mitochondria according to the results of redox balance assays under the conditions used in the present study. Previous reports demonstrated that active mitochondrial metabolism in epimastigotes leads to low level of ROS production. Three stress conditions used in the present study induce an increase in the percentage of parasites producing ROS, which is in agreement with the detected mitochondrial impairment. These results confirm the importance of combined acidic and nutritional stresses for the complete phenotype of metacyclogenesis; the former increases the number of parasites in oxidative stress, and the latter increases the electron leakage. These profile may result from complex III modulation because complex III is the main source of mitochondrial ROS in trypanosomatids [48]. However, the role of the antioxidant system in the control of

ROS production in AcS should be considered because our results suggest mobilization of different reducing molecules, such as TR and TrypP, during AcS.

Stress-independent recurrence of the autophagic phenotype in parasites was confirmed by electron microscopy that demonstrated an increase in the percentage of MDC+ and Atg8+ parasitesAtg. Autophagy is the main machinery to remodel and recycle the organelles and macromolecules in eukaryotes [15]. Certain molecular components of this pathway were identified in trypanosomatids, and Atg8 detection was previously established as the standard method to monitor autophagic flux Atg[13,20,49]. Based on the previous bioinformatics analysis [18], the qPCR targets were selected considering the involvement of autophagy: TOR1 and TOR2 (induction and signaling), Atg3, 4, 7, and 8 (vesicle expansion and completion), Atg15 (vesicle breakdown), and Atg9 and 18 (retrieval). Interestingly, AlkS increased the levels of the transcripts of the components of all four tested steps confirming that autophagy is exacerbated at both time points. In LoFBS and AcS, the transcripts of TOR and Atg genes were overexpressed at two time points. These data are in agreement with a decrease in parasite viability. Importantly, the biological role of TOR in trypanosomatids is unclear. In *T. brucei*, rapamycin increases the number of autophagosomes by inhibiting TOR2 and does not influence TOR1 [21,50]. In *T. cruzi*, mechanistic properties of TOR have not been investigated and deserve further studies.

Few studies have aimed to describe the biological role of autophagy in trypanosomatids. Alvarez and colleagues (2008) investigated the role of this pathway in *T. cruzi* metacyclogenesis and demonstrated that Atg8.1 is expressed in autophagosomes during starvation [20]. *In silico* approaches identified that four Atg4 isoforms in other trypanosomatids control the dynamics of four Atg8 isoforms [51–53]. In LoFBS and AcS, a pronounced exacerbation of autophagy monitored by Atg8 expression is detected at the initial time point and is maintained up to 96 h. Epimastigotes incubated in the TAU medium for 2 h were reported to have a strong

autophagic phenotype suggesting that the induction by a combination of acidic and nutritional stresses potentiates autophagy during metacyclogenesis [20,39,54]. The importance of autophagy in other *T. cruzi* differentiation processes, such as amastigogenesis, was also postulated reinforcing the hypothesis that autophagy is essential for parasite success in distinct hosts [39,54].

Currently, only macroautophagy identified by autophagosomes was described in protozoa. However, our ultrastructural data suggest the existence of microautophagy at least in 96 h. In the literature, microautophagy is defined by invagination of the lysosomal membrane engulfing the cellular material to be degraded [13]. The present study demonstrated invagination of the reservosomes membrane, and the micrographs suggest various stages of this process culminating in the formation of small vesicles inside the organelle. However, due to the absence of specific microautophagy markers, this process requires additional investigation.

Autophagy is involved in organelle remodeling, which was exacerbated in combination with mitochondrial impairment (functional and morphological) in epimastigotes under pH and nutritional stress conditions. ROS-induced autophagy and/or mitophagy may be a survival strategy in stressed parasites. The interplay between mitochondrion and autophagy has been reported under various biological conditions in higher eukaryotes, including cells under nutritional amino acid stress. a reduction in mitochondrial respiration leads to the activation of cAMP-dependent protein kinase A (an autophagy regulator) that negatively regulates mitochondrial biogenesis and reducing mitochondrial activity [55,56]. In *T. brucei*, glycosomes are degraded by selective autophagy, and its process is correlated to the parasite bioenergetics [22]. At present, correlation between mitochondrial metabolism and autophagy was not suggested in *T. cruzi.* To evaluate the interference of autophagy on ROS production triggered by nutritional and pH stresses, the autophagic inhibitor 3-MA was added before the stress induction, and H_2O_2 generation was measured by the Amplex Red method. Interestingly, inhibition autophagy resulted in an increase in

ROS levels in parasites subjected to AlkS similar to the phenomena previously described in other cellular models [57] suggesting that a reduction in autophagy can be involved in stimulation of oxidative stress. The role of autophagy in the turnover of damaged organelles and other cellular structures is documented in almost all eukaryotes and can be a crucial step for epimastigote survival in triatominae rectum in the first hours after the insect blood meal when alkaline excretion creates an important chemical barrier [38] to limit the parasite population. PI3K inhibitors, such as 3-MA, are extensively used to inhibit autophagy [13]; however, PI3K signaling regulates various cellular processes suggesting that these data require careful confirmation by other methods.

In another set of experiments, NAC was added before stress induction, and the Atg8 expression was assayed. Previous studies demonstrated that under physiological conditions, moderate levels of ROS can serve as a signal in several signaling pathways, including autophagy [58–60]. However, stress conditions, including nutrient deprivation, increase ROS production and consequently exacerbates autophagy [61,62]. According to our data, mitochondrial dysfunction caused by stress conditions induces an increase in ROS levels that may lead to a random cellular damage resulting in activation of the autophagic machinery.

In conclusion, nutritional deprivation and pH variations induce mitochondrial dysfunction and autophagic activity with a direct correlation between these two phenotypes. At short exposure, acid stress and nutritional deprivation lead to phenotypes characteristic for metacyclogenesis, including mitochondrial remodeling and autophagy. However, the absence of a combination of the stresses and specific stimuli prevents complete differentiation. Since alkaline stress is a transient condition in parasite life cycle due to blood digestion, medium alkalization triggers a survival mechanism to increase the autophagy flux to recycle cellular structures and decrease oxidative damage. A summary of the observed effects induced by stress conditions on *T. cruzi* is schematically depicted in Fig. 7. Additional detailed analysis is essential

to identify the cellular, biochemical, and molecular mechanisms involved in *T. cruzi* adaptation to environmental conditions to develop efficient strategies of blocking of parasite differentiation.

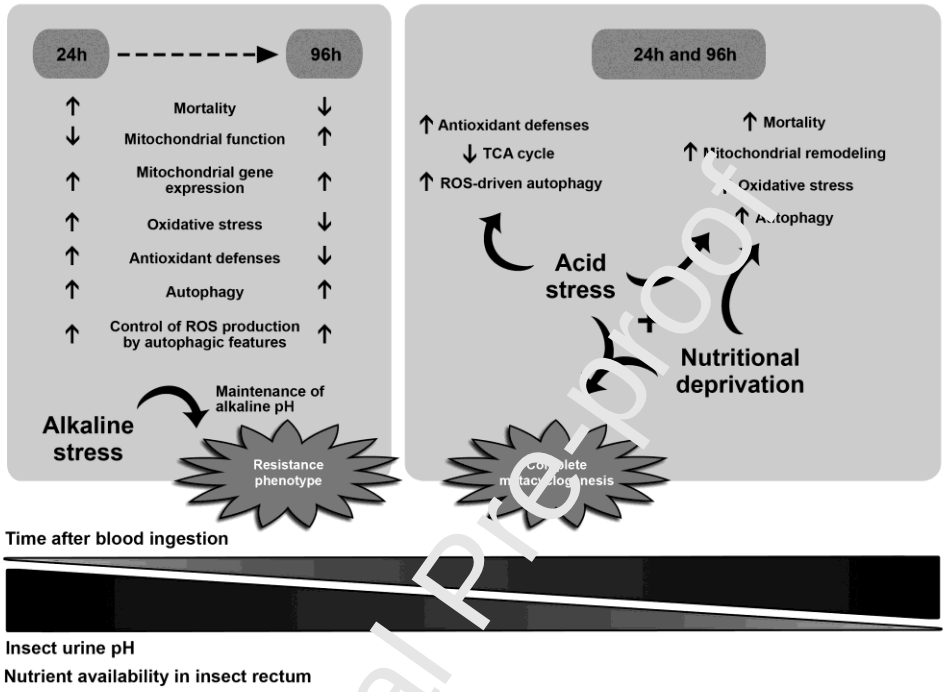


Fig. 7. Mechanistic assumptions about pH stress and nutritional restriction in *T. cruzi* epimastigotes. The parasite exposed to alkaline condition presented early mitochondrial dysfunction and increased autophagy, recovered by the time, a suggestive of resistance phenotype. On the other hand, acid stress or nutritional deprivation led to remarkable mitochondrial remodeling, ROS production and autophagy exacerbation in 24h, maintained up to 96h. The combination of low pH and low availability of nutrients triggers the metacyclogenesis at this period. Our data suggest that autophagy and the mitochondrial plasticity and remodeling as a crucial component for the success of the parasite differentiation process.

Credit authorship contribution statement

RMB: conceived the study, planned experiments; performed experiments; analysed data; provided financial support, confirmed the accuracy of data presented in the manuscript and wrote the manuscript; YPR.: performed

experiments; analysed data; wrote the manuscript; MF: performed experiments, analysed data; CMR: performed experiments; RS: performed experiments; ACB: performed experiments; analysed data and wrote the manuscript; NP: perfomed experiments; PC: validated anti-Atg8 antibody; SLDC: provided financial support.

Funding

Funding was provided by Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Oswaldo Cruz (FIOCRUZ).

Declaration of Competing interest

All authors declare that they have no financial or other competing interests.

Acknowledgments

We would like to thank Drs. Adeilton Brandão, Renata Gonçalves, Luiza Pereira and Vitor Ennes-Vidal for his support in primers design and molecular analysis, and Dr. Pedro Lagerblad for open his lab to our qPCR and part of enzymatic analysis.

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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